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Spilanthes acmella ethanolic flower extract: LC–MS alkylamide profiling and its effects on sexual behavior in male rats

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ABSTRACT

According to Indian Systems of Medicine. Spilanthes acmella (L.) Murr. (Family - Asteraceae), is considered effective in the treatment of sexual deficiencies especially due to ageing. In the present study, characterization of ethanolic extracts of the Spilanthes acmella flower and its effect on general mating pattern, penile erection and serum hormone levels of normal male Wistar albino rats were investigated and compared with sildenafil citrate. In vitro nitric oxide release was also investigated in human corpus cavernosum cell line. As N-alkylamides are a promising group, their profiling was performed using a gradient reversed phase high performance liquid chromatography/electrospray ionization ion trap mass spectrometry (HPLC/ESI-MS) method on an embedded polar column. MS¹ and MS² fragmentation data were used for identification purposes. For assessment of sexual behavior, animals were divided into five groups of eight male rats. The extracts (50, 100 and 150 mg/kg body weight/day) and sildenafil citrate (5 mg/kg body weight/day) (positive control) were administered orally for 28 days. The behavioral and sexual parameters were observed at days 0, 15, 28 and after a lapse of 7 and 14 days of discontinuance of drug treatment. Five N-isobutylamides, one 2-methylbutylamide and one 2-phenylethylamide were identified. The orally administered extract had a dose dependent positive effect on mounting frequency, intromission frequency and ejaculation frequency and the most significant effects (p < 0.05) were observed at 150 mg/kg treatment, even after a lapse of 7 and 14 days of discontinuance of drug treatment. A dose dependent effect was also observed on the FSH, LH and testosterone serum levels. With 150 mg/kg of ethanolic extract the values for FSH, LH and testosterone were 3.10 ± 0.25 mlU/ml, 6.87 ± 0.18 mlU/ml and 3.72 ± 0.12 ng/ml, respectively. In vitro nitric oxide release was 21.7 ± 2.9 μ M, which was significantly higher compared to the control group (p < 0.01). Sildenafil citrate exhibited also a significant effect on NO release, but no effect on hormone levels of rats was observed. The aphrodisiac potential of an ethanolic Spilanthes acmella extract was demonstrated in vitro and in vivo. N-Alkylamides might attribute to the improved sexual potential. Study lends support to the traditional utilization of S. acmella as a sexual stimulating agent.

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Introduction

Human search for sexual enhancers from natural substances is as old as civilization itself. Ancient history in most cultures helped society in its desire to improve the sexual experience as evident by writings in holy texts and sculptures in Hindu temples. For many years, people have searched for ways to achieve sexual desire, sexual health and sexual techniques (Jain et al. 2010). Sexual function is an important component of quality of life and subjective well-being in humans. Sexual problems are widespread and adversely affect mood, and interpersonal functioning. The main problems are related to sexual desire and male erectile dysfunction. Successful treatment of sexual dysfunction may improve not only sexual relationships, but also the overall quality of life (Shin et al. 2010).

Sexual dysfunctions increase with ageing and etiological factors, including degenerative diseases, increase in injuries and stress associated with industrialized lifestyles. It can be treated by both medical and surgical modalities. However, plant-derived and herbal remedies continue to be a popular alternative (Rowland and Tai 2003). For several hundred years, people around the world have



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used locally grown plants as supplements to energize, vitalize, and eventually to improve male sexual functions.

The availability of the large number of sex-improving drugs in the traditional Ayurvedic System of Medicine is a unique and distinctive feature of this system. In this system, Rasayana drugs constitute a special category, the uses of which are advocated for rejuvenation, revitalization and longevity. A special class of Rasayana drugs is known as Vrishya or Vajikarana. They are associated with an improvement of male sexual potency and thereby ensure a *supraja*, or better progeny. Traditionally, the main aim of using Vajikaran was to achieve successful copulation for healthy reproduction, along with an improvement in sexual pleasure as an additional benefit. Vajikaran drugs are specially recommended to people suffering from sexual insufficiency and people in advanced age losing interest in sexual act or failing in sexual performance (Sharma et al. 2010). Besides having many specific drugs for enhancing sexual functions, the most commonly used is "akarkara". Different plants are being referred to as akarkara, but the most prominent one is Spilanthes acmella (S. acmella). The akarkara plants are empirically used as powerful aphrodisiac in traditional medicine practice in cases of sexual debility or depressed desire.

S. acmella has long been used in the traditional Indian Systems of Medicine for the treatment of various sexual inadequacies and is claimed to improve sexual functions in man. Other ethnopharmacological claims associated with S. acmella include its usage in treatment of rheumatism, inflammation, stimulant and as sialagogue for stammering, tongue paralysis, stomatitis, toothache, headache and treatment of asthma, rheumatism, fever, sore throat and hemorrhoids and gum infections (Prachayasittikul et al. 2009; Vijevaanandhi et al. 2007). In addition, its extract is traditionally added to nutritional supplement and cosmetics to accelerate repair of wrinkles which are caused due to vasoconstriction of skin. Pharmacologically, S. acmella is a potential vasodilator, antioxidant (Wongsawatkul et al. 2008) and immunomodulator (Savadi et al. 2010). Previous studies have demonstrated its diuretic, antibacterial, and anti-inflammatory activities (Ratnasooriya et al. 2004). Recently, it has also been demonstrated that spilanthol in S. acmella extracts permeates the skin and buccal mucosa (Boonen et al. 2010a.b).

Phytochemical analysis of ethanolic extract of *S. acmella* revealed that it is rich in *N*-alkylamide. The main *N*-alkylamide of *Lepidium meyenii* (Maca), known as macamide, and of *Anacy-clus pyrethrum*, known as pellitorine, have been found effective in improvement of sexual behavior (Cicero et al. 2001; Sharma et al. 2010). Keeping in view the growing popularity and market interest in herbs for sexual problems, and lack of scientific studies on *S. acmella*, present investigation was undertaken to evaluate the scientific foundation for the concept of Vajikaran Rasayana.

Materials and methods

Animal stock

The protocol for experimentation was approved by Institutional Animal Ethics Committee of Dr. Hari Singh Gour University, Sagar, India (Animal Eths Comm/IE/98/Reg No379/01/ab/CPCSEA) and was in accordance with international standard on the care and use of experimental animals. Inbred, 40 sexually active Wistar strain male albino rats, weighing 150–180g were used for the present study. Female rats from the same strain rats, used as stimulus for evaluation of sexual behavior, were prepared for experimentation, using the method reported by Agmo (2003). In brief, before all testing sessions, female estrus was induced by administration of estradiol benzoate (25 μ g/rat), followed by progesterone (250 μ g/rat), 48 h later. Females were used between 4 and 8 h after the progesterone administration. Both steroids were purchased from Sigma (St. Louis, MO, USA). They were dissolved in arachis oil (Kriti, India) and injected subcutaneously in a volume of 0.1 ml/rat. The rats were housed at room temperature ($24 \pm 2 \degree$ C) on a reversed day-night cycle (dark from 06:00 to 18:00) and relative humidity of 50–55%. They were fed with a standard pellet diet and water *ad libitum*.

Preparation of extracts

The plant *S. acmella* was identified and authenticated at Department of Botany, Dr. H.S. Gour University Sagar (M.P.) India and a herbarium has been deposited there as well (Herbarium No. Bot/02-8/2008). The flowers of the plant were collected from the area in vicinity of the campus and were dried at room temperature (25–35 °C). Next, the flowers were reduced to powder and passed through a sieve (60 mesh), fed in a soxhlet extractor and extracted with ethanol (95%) till complete exhaustion. The extract was collected and dried under vacuum by using a rota vapor (Heidolph, Germany). The yield of the ethanolic extract was found to be 6.1% (w/w). Before oral administration, the *S. acmella* extract was suspended in 1% sodium CMC.

HPLC/ESI-MS analytical N-alkylamide profiling

Analytical samples were prepared by dissolving the extract and reference material in a acetonitrile:water mixture (50/50, v/v), followed by filtration over a 0.45 µm nylon HPLC filter (Whatman, Dassel, Germany). The LC/MS apparatus consisted of a Spectra System SN4000 interface, SCM1000 degasser, P1000XR pump, AS3000 autosampler and was equipped with a Finnigan LCO Classic ion trap mass spectrometer in positive ion mode (all Thermo, San José, CA, USA). Data were acquired using Xcalibur 2.0 software (Thermo, San José, CA, USA). A Prevail RP C₁₈ column (250 mm \times 4.6 mm, 5 μ m) with suitable guard cartridge $(4.6 \text{ mm} \times 7.5 \text{ mm}, 5 \mu \text{m})$ (both from Grace, Lokeren, Belgium) was used (Boonen et al. 2010b). The injection volume was 25 µl. The flow rate was set to 1.0 mL/min and the linear gradient used was as follows (where A = 1% acetic acid in ultrapure water and B = HPLC grade acetonitrile): $t = 0 \min$, A:B (80:20, v/v); t=0-150 min, A:B (10:90, v/v); t=150-151 min, A:B (80:20, v/v); *t* = 151–166 min, A:B (80:20, v/v). ESI was conducted using a capillary voltage of 3 kV. Nitrogen was used as the sheath and auxiliary gas with the heated capillary set at 275 °C. MS-MS spectra were obtained by collision induced dissociation (CID) of the parent m/z, with the relative collision energy set at 35%. Identification was based on the m/z values and fragmentation ions, while quantification was performed using a laboratory reference material ("A. Vogel Spilanthes", Biohorma, Lummen, Belgium) with spilanthol (0.11%, w/w) as the reference biomarker.

Treatment

Male rats were divided into five equally spaced groups. Group I served as blank and received only vehicle, *i.e.* normal saline. Groups II, III and IV were given an oral daily dose of 50, 100 and 150 mg/kg *S. acmella* extract, respectively. Group V rats were administered daily and orally with 5 mg/kg sildenafil citrate (a generous gift of Sun Pharma, India). The course of the treatment was 28 days.

Toxicity study

Two groups, containing six male and six female rats were used. The test group received an oral dose of 2 g/kg *S. acmella* extract. The rats in the control group received 1 ml of tap water. Behavioral parameters such as convulsions, sedation, and hyperactivity, grooming and accelerated breathing were observed. The animals were observed continuously for any behavioral changes or death for 1 h, then intermittently at 6 h, 24 h and finally 48 h after drug administration.

Functional studies

Penile erection

Penile erection was determined by using the method reported by Thakur et al. (2009). In brief, each male rat was placed in a transparent plexi glass cabin $(60 \text{ cm} \times 40 \text{ cm} \times 40 \text{ cm})$ that was divided in half by 2 sheets of plastic fiber mesh, preventing contact but allowing auditory, visual, and olfactory stimuli. Ventral as well as lateral viewing and recording of the whole experimentation was facilitated by appropriately placing a mirror. After a 5-min adaptation period, the test was started by placing an estrus female on the other side of the cage. Cages were cleaned before shifting the animals of different groups. The number of erections were recorded and tabulated. Erection in rats was marked by the visibility of the penis out of its sheath or by grooming of the penis, which is another indicator of penile erection in rats. Penile erection index (PEI) was calculated as per the methodology reported by Islam et al. (1991), i.e. by multiplying the percentage of rats exhibiting at least one episode of penile erection during 30-min observation period with the mean number of penile erections: PEI = percentage of rats exhibiting penile $erection \times mean$ number of erections.

Parameters for sexual behavior analysis

The experimentation began by switching off the light at 08:00 A.M. After an interval of 20 min past turning off the light, the experimentation room was lit with a dim red illumination. The male rat was placed in a rectangular plexi glass chamber. After about 10 min, when the rat was acclimatized to the chamber condition, a sexually receptive female rat was dropped silently from one side of the chamber as stimulus. The observations in the 30 min for sexual behavior were recorded for the following parameters (Thakur et al. 2009; Chauhan et al. 2009).

Mounting behavior: Mount frequency (MF) was determined by counting the number of pre-ejaculatory mounts with and without intromission in given period of observation. Mount latency (ML) was calculated as the time lapse from the introduction of female to the occurrence of first mount.

Intromission behavior: Intromission latency (IL) was calculated as the time when first intromission was observed after introduction of female in the cage. Intromission frequency (IF) was considered as total number of (pre-ejaculatory) intromission within 30 min.

Ejaculation behavior: Ejaculation frequency (EF) was calculated as the number of ejaculations observed in the 30 min period and Post-ejaculatory Interval (PEjI) was considered as the time interval between ejaculation and the first mount of the 30 min period.

Effects of drug after withdrawal of treatment

Daily dosing of the rats was stopped after 28 days of the treatment. In continuation, the observations of different parameters connoting sexual behavior were made on the 7th and 15th day after withdrawal of the treatment of respective groups (Sharma et al. 2010).

Serum FSH, LH and total testosterone hormone measurements

On the 28th day, blood was collected to measure serum FSH, LH and testosterone levels. Blood samples were spun at $2500 \times g$ for 10 min in a table top centrifuge. The serum samples obtained were analyzed to determine the concentration of FSH, LH and testosterone. Serum FSH and LH was measured (Erba Fertikit, Germany), according to the protocol provided with each kit. Serum concentration of total testosterone was measured by using a double antibody ELISA kit (Eiagen Testosterone kit, Italy). The protocol used for the

testosterone determination was according to the method described for the kit. Serum concentrations of hormones were determined in triplicate samples (Chauhan and Dixit 2010).

In vitro nitric oxide activity

DS-1 cells (human corpus cavernosum cell line) were routinely cultured in a humidified 5% CO_2 –95% air incubator in Dulbecco's modified eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 20 μ M L-glutamine, 1 mM pyruvate, 200 U/ml penicillin G, 200 μ g/ml streptomycin and 4.5 mg/ml of D-glucose at 37 °C. Cells were allowed to attach to the bottom of a 96-well plate overnight and were then exposed to the extract (10 mg/ml PBS) or placebo PBS (control) for 24 h. The cell media was assayed for the concentrations of nitrate (NO₃⁻) and nitrite (NO₂⁻), the final *in vivo* products of NO, using a commercial nitrate reductase preparation and Griess reagent kit (Cayman Chemical, USA) with the resultant absorbance being read at 550 nm using a VictorXTM plate reader (Perkin Elmer USA) (Thakur et al. 2011).

Statistical analysis

Results are expressed as mean \pm standard error mean (S.E.M.). The groups were compared by ANOVA, followed by Dunnet's test. All the statistical analysis was carried out using Instat version, 2.1 software. Etholog 2.1 was used for computation of the behavioral parameters.

Results

LC-MS analytical N-alkylamide profiling

The identification of the *N*-alkylamides in the *S. acmella* extract was performed using the experimentally obtained m/z and their fragmentation pattern (Boonen et al. 2010b). LC–MS revealed seven *N*-alkylamides in the ethanolic extract: five *N*-isobutylamides (IBA), one 2-methylbutylamide (MBA) and one 2-phenylethylamide (PEA). The total ion MS chromatogram is presented in Fig. 1. The most abundant *N*-alkylamide in the investigated *S. acmella* extract was (2*E*,4*E*,8*Z*,10*Z*)-*N*-isobutyl-



Fig. 1. Detailed TIC-MS chromatogram of the identified *N*-alkylamides in the ethanolic *S. acmella* extract [1: (2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide, 2: (2*Z*)-*N*-phenethyl-2-nonene-6,8-diynamide, 3: (2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide, 4: (2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide, 5: (2*E*,7*Z*)-*N*-isobutyl-2,7-decadienamide, 6: (2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide, 7: (2*E*,4*E*,8*Z*,10*Z*)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide].

Table 1Detected N-alkylamides in the ethanolic S. acmella extract.

Peak no.	$R_{\rm t}({\rm min})$	Precursor ion	Fragment ions	Nomenclature	Structure	Content (%, w/w)
1	56.9	230	_101· _99· 73· _56	(2F 47)-N-isobutyl-2 4-undecadiene-8 10-divnamide		0.25
1	50.5	230	-101, -33, 73, -30	(22,42) Wisobatyi 2,4 undecadene 0,10 alynamide		0.25
2	59.1	252	-149; -147; -121; -104	(2Z)-N-phenethyl-2-nonene-6,8-diynamide		0.11
3	64.2	222	-1151019973	(2E 6Z 8E)-N-isobutyl-2 6 8-decatrienamide	H ₃ C	0.20
5	0 112		110, 101, 00, 70	(22,02,02) ··· 0002431 2,0,0 accartenaniae	0	0120
4	70.8	258	-101;-99; -73; -56	(2E,7Z)-N-isobutyl-2,7-tridecadiene-10,12-diynamide	HC HC	0.12
					o II	
5	72.2	224	-101; -99; -73; -56	(2E,7Z)-N-isobutyl-2,7-decadienamide	H ₃ C NH CH ₃ CH ₃	0.04
C	72.0	226	115, 110, 07, 70, 40	(2007.07) N (2 methodische 1) 200 desetzionerside	H ₃ C	0.07
U	12.0	230	-115, -115, -87; -70; -42	(<i>2E</i> ,0 <i>Z</i> ,0 <i>E</i>)- <i>I</i> V-(<i>Z</i> -IIIEIIIYIDULYI)- <i>Z</i> ,0,8-decalifeifailiide	0	0.07
					H ₃ C	
7	77.6	248	-99; -73; -56	(2E,4E,8Z,10Z)-N-isobutyl-dodeca-2,4,8,10-tetraenamide	ĊH ₃	0.71

Table 2	
Effects of ethanolic extracts of S. acmella on penile erection index (PEI) in male rats.	
	-

Group	Days of treatment			Days after withdrawal treatment	
	0	15	28	7	15
Control	23.9 ± 4.51	22.1 ± 2.40	23.9 ± 3.10	27.9 ± 3.39	27.6 ± 4.15
EE 50	21.2 ± 2.60	27.9 ± 2.61	38.81 ± 2.51	36.7 ± 4.40	30.4 ± 4.50
EE 100	21.1 ± 1.90	33.21 ± 7.01	$63.8 \pm 6.7^{**}$	$61.4 \pm 6.6^{**}$	$47.15 \pm 5.61^{*}$
EE 150	23.0 ± 2.20	$44.4 \pm 5.50^{**}$	$69.5 \pm 7.9^{**}$	$69.5 \pm 7.6^{**}$	$63.8 \pm 7.62^{**}$
SC	23.9 ± 1.10	$80.5 \pm 6.69^{**}$	$94.1 \pm 5.51^{**}$	27.56 ± 5.32	22.10 ± 2.90

* *p* < 0.05.

^{**} *p* < 0.01.

dodeca-2,4,8,10-tetraenamide (0.71%, w/w), while spilanthol was present at 0.20% (w/w). Table 1 depicts the retention time of each of the present *N*-alkylamides, their precursor ion which corresponds to the molecular mass +1 (M+H⁺/Z with Z=1) and characteristic fragmentation information (formed by CID), as well as their proposed structure.

Toxicology

No acute toxicity of ethanolic extract was demonstrated; no mortality or changes in behavior were observed in the control group or in the group of male and female rats treated with the *S. acmella* extract.

Penile erection

The administration of the extract resulted in a significant dosedependent increase in PEI as compared to control group (Table 2). The PEI was nearly three times higher in the ethanolic extract dosed 150 mg/kg body weight (EE 150) and four times higher in the sildenafil citrate treated group during the treatment period.

Sexual behavior

The observations of the sexual behavior study are illustrated in Figs. 2–7 while the numerical data are given in Table A1. Treatment of the animals with the ethanolic *S. acmella* extract brought a remarkable change in the sexual behavior. A decrease in ML, IL

Table 3

inects of ethalione extracts of 5, ucment on 1511, En and testosterone in male rats.	Effects of ethanolic extracts of S. acmella on FSH, LH and testosterone in male r	ts.
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Group	FSH (mlU/ml)	LH (mlU/ml)	Testosterone (ng/ml)
Control	1.41 ± 0.09	4.63 ± 0.11	1.73 ± 0.11
EE 50	1.73 ± 0.18	4.87 ± 0.10	$2.07 \pm 0.07^{*}$
EE 100	$2.52\pm0.27^{**}$	$5.23 \pm 0.21^{*}$	$2.91 \pm 0.09^{**}$
EE 150	$3.10 \pm 0.25^{**}$	$6.87 \pm 0.18^{**}$	$3.72 \pm 0.12^{**}$
SC	1.42 ± 0.41	4.60 ± 0.15	1.71 ± 0.13
-			

* *p* < 0.05.

** p<0.01.

and PEjI and increase in MF, IF and EF were observed, in a dose dependent manner. MF and EF were significantly altered in the treatment groups on the 15th and 28th day and 7 and 15 days after treatment withdrawal, whereas sildenafil citrate altered these parameters only on the 15th and 28th day of the treatment but not after withdrawal of the treatment.

Serum LH, FSH and testosterone levels

Compared to the control group, administration of 100 and 150 mg/kg body weight *S. acmella* extract for 28 days had significant effect on LH and FSH concentration in the serum (Table 3). In addition, serum testosterone level also increased significantly (p < 0.05) in animals who received 50, 100 and 150 mg/kg *S. acmella* extract *in* comparison to the control group (Fig. 8). No effect of sildenafil citrate was observed on the hormone levels in rat sera.



Fig. 2. Effects of ethanolic extracts of *S. acmella* on mount latency in male rats. All values are expressed as mean ± S.E.M., *n* = 8; **p* < 0.05 and ***p* < 0.01 considered significant as compared to control.



Fig. 3. Effects of ethanolic extracts of *S. acmella* on mount frequency in male rats. All values are expressed as mean ± S.E.M., *n* = 8; **p* < 0.05 and ***p* < 0.01 considered significant as compared to control.



Fig. 4. Effects of ethanolic extracts of *S. acmella* on intromission latency in male rats. All values are expressed as mean ± S.E.M., *n* = 8; **p* < 0.05 and ***p* < 0.01 considered significant as compared to control.



Fig. 5. Effects of ethanolic extracts of *S. acmella* on intromission frequency in male rats. All values are expressed as mean ± S.E.M., *n* = 8; **p* < 0.05 and ***p* < 0.01 considered significant as compared to control.

In vitro nitric oxide release

Nitric oxide release was evaluated in DS-1 cell line and the relative % enhancement in nitric oxide release was compared to the solvent control ($8.83 \pm 0.98 \,\mu$ M). The *S. acmella* extract ($10 \,\text{mg/ml}$) exhibited a relative nitric oxide release of $21.7 \pm 2.9 \,\mu$ M as compared to a nitric oxide release of $35.42 \pm 2.7 \,\mu$ M with sildenafil citrate. The results are suggestive for the involvement of nitric oxide release mechanism for observed improvement in PEI by the *S. acmella* extract.

Discussion

Androgens have long been known to have a major stimulatory influence on several aspects of male sexual behavior, including penile erection. Sexual behavior is dependent on normal



Fig. 6. Effects of ethanolic extracts of *S. acmella* on ejaculation frequency in male rats. All values are expressed as mean ± S.E.M., *n* = 8; **p* < 0.05 and ***p* < 0.01 considered significant as compared to control.



Fig. 7. Effects of ethanolic extracts of *S. acmella* on post-ejaculation latency in male rats. All values are expressed as mean ± S.E.M., *n* = 8; **p* < 0.05 and ***p* < 0.01 considered significant as compared to control.

functioning of the hypothalamo-pituitary-gonadal axis. In most mammalian species studied, castration has been found to decrease substantially the erectile responses to a variety of stimuli, whereas androgen replacement reversed these effects (Andersson 2001).

Testosterone (T) is the main male gonadal hormone produced by the interstitial cells of the Leydig in the testis. T also helps in maintaining body shape, and increasing muscle mass and strength. The increase in testosterone should enhance androgen-dependent parameters such as mating behavior and maintenance of spermatogenesis. FSH stimulates spermatogenesis in the Sertoli cells and LH (ICSH) stimulates synthesis and release of testosterone in the Leydig cells (Chauhan and Dixit 2010). Testosterone may also facilitate male sexual behavior by increasing dopamine release in the medial preoptic area and potentiating nitrergic neurotransmission (Hull et al. 1999; Putnam et al. 2001).

Present findings provide experimental evidence that the ethanolic extract of *S. acmella* flowers, used as a traditional medicine, possesses aphrodisiac properties. We demonstrated that the oral administration of its ethanolic extract was able to improve sexual performance, particularly of sexual arousal in male rats and



Fig. 8. Effects of ethanolic extracts of *S. acmella* on serum FSH, LH and testosterone level in male rats. All values are expressed as mean ± S.E.M., *n* = 8; **p* < 0.05 and ***p* < 0.01 considered significant as compared to control.

that it promotes the expression of male sexual behavior. Compared with the controls, the percentages of mounting and intromission in rats were significantly increased in animals by administration of the extract. Importantly, the extract has the capacity to produce longterm effects after even 14 days past cessation of treatment. The reduction of ML, IL and PEJI is generally suggested to be indicative of an improved copulatory behavior, particularly when observed together with an increase in mounting and ejaculating animals (Bitran and Hull 1987).

The ability of different medicinal plants to improve sexual function as illustrated in the case of Tribulus terrestris, Panax ginseng, Ferula hermonis was ascribed to increased levels of testosterone in the serum (Zanoli et al. 2009). In another study, the effect of GnRH peptides substitution of Gly-NH2 with alkylamides maintained (methylamide and ethanolamide) or increased androgen activity up to 600% (propylamide and ethylamide) whereas substitution with larger amides (pyrolidineamide and morpholineamide) or D-amino acids decreased activity (Sealfon et al. 1997). It is possible that the alkylamide present in the extract may have an effect on androgen. There is a possibility of developing the ethanolic extract of S. acmella flowers as a therapeutic principle for stimulating male sexual activity, especially in cases where there are moderate sexual deficiencies. The presence of spilanthol and other alkylamides in the extract may be suspected as possible contributor to the observed effect of improved sexual function. The basis for such a premise is drawn from the libido enhancing properties of alkylamides isolated from the roots of Lepidium sativum. Oral administration of a purified lipidic extracts from Lepidium meyenii and Anacyclus pyrethrum increased intromissions and mounts frequency in normal mice (Zheng et al. 2000; Sharma et al. 2010). In our study, ethanolic extract treated rats also showed increased intromissions and mount frequency. It is likely that alkylamide may mimic the action like testosterone or stimulate secretion of testosterone that improves sexual behavior.

Enhanced erectile function is directly correlated to enhanced sexual performance in most cases (Fink et al. 2002). Penile erection is under direct control of NO release via neuronal, endothelial and inducible nitric oxide synthase activity (Burnett et al. 1992). The enhanced PI activity *in vivo* of the extracts is directly correlated with the inducible NO (*i*NO) activity *in vitro* by the extracts of the drug, and is suggestive of improved erectile response and enhanced sexual pleasure, which are desirable attributes of any aphrodisiac agent. The present study also provides a good correlation between the *in vivo* improvement of penile erection and NO releasing activity of the extracts.

There was no toxic effect observed in the rats administered with these dosages of *S. acmella* flower extract in the present study. In fact, no effects were seen even at higher dosages as well. The exact molecular mechanisms of the different *N*-alkylamides are not yet fully elucidated. It appears that the tingling effect of *e.g.* α -sanshool may be mediated by different ion-channel receptors on different types of sensory neurons, like the capsaicin (transient receptor potential vanilloid type 1 (TRPV1)), TRPA1, TRPM8 receptor, while more recently, emphasis is placed on distinct receptors like KCNK3, KCNK 9 and KCNK 18 (two-pore potassium channels) (Bautista et al. 2008). However, there is a consensus that several mechanisms are involved, with the relative importance dependent on the *N*-alkylamide structure (Viane 2011).

Further investigations are in progress to ascertain the mechanism by which chemical constituents in *S. acmella* flowers ethanolic extract exerts potent aphrodisiac activity in males. The effect of the drug on female sexual behavior and male fertility remains to be studied.

In conclusion, the present study provides evidence that the ethanolic extract of *S. acmella* flowers is a potent stimulator of sexual behavior in male rats. There was an overall increase in the

sexual behavior parameters for the treated groups, as reflected in the increasement of MF, IF and EF, and reduction in ML, IL and PEJI. These results were statistically significant even after discontinuing the treatment. The effect persisted, suggesting encapacitation of the body for improved sexual activity. It is interesting to note that sildenafil citrate improved the sexual parameters only during the treatment and was ineffective after its withdrawal. Based on our observations, *S. acmella* flowers ethanolic extract can be considered to possess aphrodisiac properties.

Conflict of interest

No conflict to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2011.06.001.

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Table AI: Effects of ethanolic extracts of S. acmella on sexual behavior in male rats.

Sexual behavior	Groups	0 dav	15-day	28-day	7 days after withdrawal	15 days after withdrawal
parameter	oroups	o unj	treatment	treatment	treatment	treatment
	Control	170 ± 10.02	172 ± 9.15	168.6 ± 6.36	172.3 ± 4.33	174 ± 2.08
	EE 50	173 ± 13.15	162 ± 4.04	$146.3 \pm 8.39*$	$149.3 \pm 9.81*$	151.6 ± 7.26
Mount latency	EE 100	177 ± 8.10	$151 \pm 5.77*$	$129.6 \pm 7.93 **$	$130.5 \pm 5.88 **$	$133.6 \pm 8.8*$
(ML)	EE 150	174 ± 11.14	$140 \pm 9.88 **$	$99.3 \pm 4.25 **$	$108.6 \pm 6.21 **$	$110 \pm 11.5 **$
	SC	172 ± 17.01	$130 \pm 4.61 **$	$117.6 \pm 9.71 **$	160.1 ± 3.32	163.3 ± 7.07
	Control	10.05 ± 1.05	10.12 ± 1.1	10.65 ± 1.7	11 ± 1.20	11.3 ± 0.88
	EE 50	11.14 ± 1.45	12.5 ± 1.51	17.3 ± 0.66	$18 \pm 2.15^*$	$15.3 \pm 0.76 *$
Mount frequency	EE 100	9.92 ± 2.42	15.3 ± 1.76	$20.66 \pm 2.40*$	20.1±1.15**	$17.6 \pm 0.88 **$
(MF)	EE 150	11.56 ± 1.95	$17.6 \pm 1.40 **$	$23.3 \pm 2.72 **$	$22.6 \pm 2.30 **$	$19.3 \pm 1.12 **$
	SC	10.54 ± 2.01	$20.1 \pm 1.15 **$	$22.0 \pm 2.57 **$	13.6 ± 1.80	12.3 ± 1.40
	Control	290.2 ± 22.46	285.6 ± 12.40	287.6 ± 10.91	289.3 ± 17.60	290.5 ± 19.23
	EE 50	289.6 ± 16.96	264.2 ± 19.06	$206.3 \pm 14.53 **$	$221.7 \pm 16.00 **$	241.3 ± 9.28
Intromission	EE 100	280.9 ± 11.40	255 ± 13.22	$183.3 \pm 8.9 **$	$190.0 \pm 13.5 **$	206.3±21.80*
latency (IL)	EE 150	287.4 ± 14.54	$238.3 \pm 17.26*$	150 ±12 .8**	$160.6 \pm 14.21 **$	$165 \pm 15.9 **$
	SC	283.6 ± 15.44	$186.3 \pm 13.17 **$	$176.6 \pm 8.71 **$	276.6 ± 18.19	273.3 ± 12.01
	Control	2.11 ± 0.25	2.13 ± 0.18	3.1 ± 0.44	3.26 ± 0.37	3.33 ± 0.33
	EE 50	2.00 ± 0.29	3.0 ± 0.57	$5.66 \pm 0.63*$	5.0 ± 0.55	4.66 ± 0.66
Intromission	EE 100	2.21 ± 0.33	$4.3 \pm 0.43 **$	$7.31 \pm 0.87 **$	7.04± 0.57**	$6.66 \pm 0.33 **$
frequency (IF)	EE 150	1.96 ± 0.16	$5.3 \pm 0.31 **$	$9.10 \pm 0.57 **$	$9.21 \pm 0.84 **$	$8.60 \pm 0.62 **$
	SC	2.19 ± 0.46	$7.60 \pm 1.04 **$	$8.01 \pm 1.07 **$	3.33 ± 0.80	3.52 ± 0.66
	Control	1.91 ± 0.42	1.83 ± 0.16	1.8 ± 0.20	1.81 ± 0.10	1.91 ± 0.06
Figuration	EE 50	1.85 ± 0.26	2.53 ± 0.37	2.81±0.43*	$2.83 \pm 0.38*$	2.46 ± 0.24
frequency (FF)	EE 100	1.95 ± 0.31	$2.76 \pm 0.08*$	$2.96 \pm 0.12 **$	$2.90 \pm 0.05 **$	$2.80 \pm 0.11 **$
frequency (EF)	EE 150	1.75 ± 0.19	$2.90 \pm 0.15 **$	$3.20 \pm 0.27 **$	$3.10 \pm 0.57 **$	$2.96 \pm 0.88 **$
	SC	1.84 ± 0.51	2.80 ± 0.41 **	$2.83 \pm 0.88 **$	1.83 ± 0.12	1.86 ± 0.13
	Control	562.42 ± 32.4	555.20 ± 22.4	556.00 ± 23.33	550.12 ± 23.09	552.60 ± 20.83
Dest sizevlatowy	EE 50	549.20 ± 43.9	538 ± 24.46	$445.0 \pm 18.93*$	$451.6 \pm 19.65*$	485.0 ± 14.43
rost-ejaculatory	EE 100	565.24 ± 54.5	523.6 ± 17.49	$395.3 \pm 28.54 **$	$393.33 \pm 16.67 **$	421.6±21.67*
Interval (PEJI)	EE 150	571.44 ± 32.4	$483.33 \pm 26.6*$	$268.66 \pm 22.5 **$	273.33 ± 14.53**	357.10± 14.57**
	SC	559.29 ± 41.4	$288.3 \pm 21.66 **$	$282.0 \pm 32.69 **$	528.2 ± 33.62	540.32 ± 28.86

*p<0.05

**p<0.01